Terbium Luminescence from Complexes of Angiotensin II, Small Peptides, and Amino Acids[1]

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ABSTRACT. Lanthanide ions have been successfully used as luminescent probes in several biological systems. The terbium (Tb) luminescence is greatly enhanced in Tb(III)-protein systems by non-radiative energy transfer from aromatic chromophores to bound Tb^{3+} ions. In this study, the luminescent properties of terbium have been used to monitor the metal-peptide interactions of angiotensin II, a linear octopeptide hormone (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe). The terbium luminescence was enhanced when the metal-complex was excited at either 259 nm or 280 nm (Phe and Tyr absorption bands, respectively). The results from a series of experiments with amino acids and small peptides show that the terbium luminescence from the Tb-Ang II complex is about the same as observed for a Tb-Phe complex when excited at 259 nm, but only 34% of the value of a Tb-Tyr complex excited at 280 nm. These results confirm the structural model of angiotensin II and show that the low enhancement of the terbium luminescence in peptide and amino acid complexes is due to weak binding and not poor energy transfer.

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INTRODUCTION

Angiotensin II (Ang II) is a linear octoapeptide hormone (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8) which elicits physiologic responses in target tissues by initially interacting with a membrane receptor. Metal ions have been shown to enhance the biological activity and receptor binding of the hormone. Recent results suggest that metal ions induce conformational changes in the hormone which produce a more physiologically active conformer (Schaechtelin et al. 1974). Consequently, many conformational-structure-function (for reviews see Marshall et al. 1974, Regoli et al. 1974, Peach 1977) and metal ion (Na^+ and Ca^{2+}, Schaechtelin et al. 1974, Schaechtelin et al. 1975, Blanc et al. 1978, Gunther et al. 1980; lanthanide ions, Lenkinski et al. 1978, Canada 1981, Lenkinski and Stephens 1981, Lenkinski and Stephens 1983) studies have been reported.

Lanthanide ions, especially terbium (Tb^{3+}) and europium (Eu^{3+}), have been used as luminescent probes in calcium-binding protein (Martin 1983) to determine calcium and lanthanide elements in dilute solution (Miller and Senkfor 1982), and to develop an immunoassay for gentamicin (Wilmott et al. 1984). In these cases, the emission from Tb^{3+} shows an enhancement factor of up to 10^5 when compared to the luminescence from free aqueous terbium. Lanthanide luminescence results from intraconfigurational f-f transitions. In aqueous solution, the lanthanide luminescence is of moderate strength even with high concentrations because the molar absorptivities are low. Water also quenches the emission. However, when the lanthanide ions are bound to proteins and certain other ligands they may be excited indirectly. The ions receive energy by radiationless energy transfer from an efficient donor, such as an excited aromatic chromophore. The ligands with aromatic chromophores also provide a more hydrophobic environment which reduces the quenching. Both of these factors contribute to the enormous enhancement of the lanthanide emission.

In this study, the luminescent properties of terbium have been used to monitor the metal-peptide interactions of angiotensin II. The luminescent properties of amino acid and small peptide complexes with terbium were also examined.

METHODS AND MATERIALS

Angiotensin II was purchased from Sigma Chemical Co; Amino acids and small peptides were obtained from Sigma and United States Biochemical Corp. All were used without purification. Angiotensin II (Ang II) solutions were made with 0.05 M HEPES buffer at pH 7.4; the amino acids and small peptide solutions were prepared in 0.01 M piperazine buffer at pH 6.5. High pH studies were not possible due to the limited solubility of Tb^{3+} in basic media. Terbium solutions were prepared with $TbCl_3 \cdot 6H_2O$(99.9%) obtained from Alfa Inorganics.

The concentrations of Ang II samples were determined from tyrosine ultraviolet absorption at 275 nm with a Cary 219 spectrophotometer by Varian. All of the luminescence measurements were made with an Aminco-Bowman Ratio II spectrofluorometer at room temperature using standard 1-cm^2 quartz cells.

For the titration experiment, 2.0 mL of the Ang II solution was placed in the fluorescence cell. The relative intensity was recorded after microliter aliquots of Tb^{3+} were added to the sample cell.

RESULTS AND DISCUSSION

When excited, terbium ions phosphoresce, producing a characteristic green emission. The phosphorescence spectrum is composed of four narrow bands from 450 to 650 nm. The most intense band (λ_{max}545 nm) is due to a $^5D_4 \longrightarrow {}^7F_5$ transition. Upon direct excitation, the most intense band in the excitation spectrum is at 260 nm, when the pH is low. However, the excitation spectrum is pH dependent, as shown in Figure 1 for a 23 mM solution of Tb^{3+}. The solution at pH 8.4 was cloudy; at pH 10, the Tb^{3+} was extensively precipitated as the hydroxide. The shift in the excitation spectrum coincides with hydroxy complex formation of Tb^{3+} with water which occurs at pH 7 (Prados et al. 1974).

When Tb^{3+} is added to a solution of Ang II, the native tyrosine fluorescence is quenched, whereas the terbium

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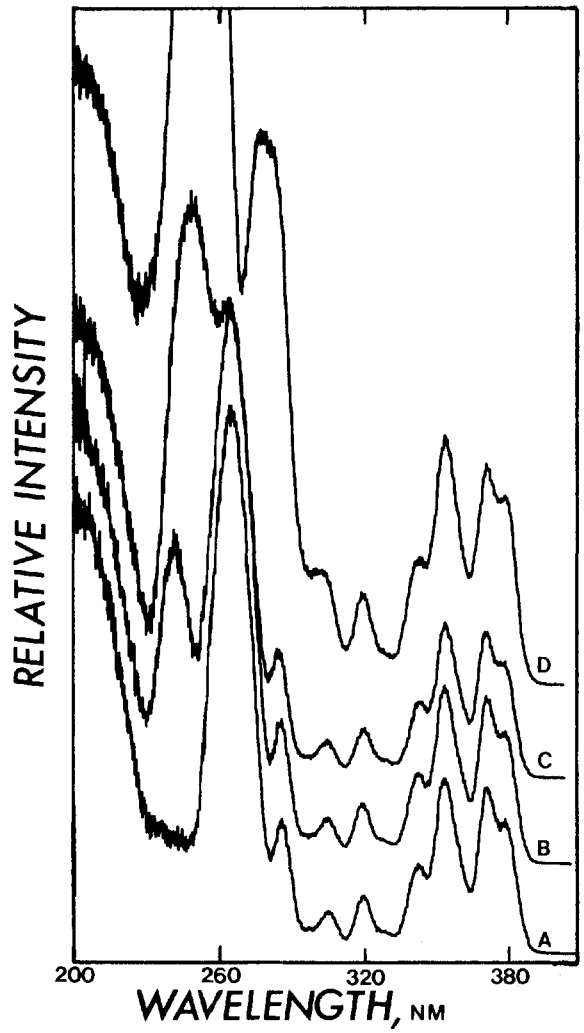

FIGURE 1. Effect of pH on the terbium excitation spectrum (emission at 545 nm). pH: (a) 6.5, (b) 7.4, (c) 8.4, (d) 10. Relative intensity shown in arbitrary units.

luminescence is enhanced. The excitation spectrum of a solution with 2.7 mM terbium is compared with the excitation spectrum of a solution with 2.7 mM terbium and 61 μm Ang II in Figure 2. The terbium emission is enhanced in the presence of Ang II. The effects of added increments of Tb^{3+} at pH 7.4 on the relative emission intensity at 545 nm, when Ang II is irradiated at 280 nm, is shown in Figure 3.

The enhancement (E) of the terbium luminescence at 545 nm is defined as:

$$E = (I_{complex} - I_o)/I_o$$

where: I_o is the relative intensity without the ligand present in solution.

The enhancement values calculated from Figure 2 are shown in Table 1. Values are reported for both tyrosine (280 nm) and phenylalanine (259 nm) excitation. The value at 280 nm is smaller than the value reported earlier (Canada 1981). The enhancement is calculated on a molar basis; the concentration of the Tb-Ang II complex in solution was determined from the dissociation constants for the carboxylates of Asp^1 and Phe^8 (Lenkinski and Stephens 1983).

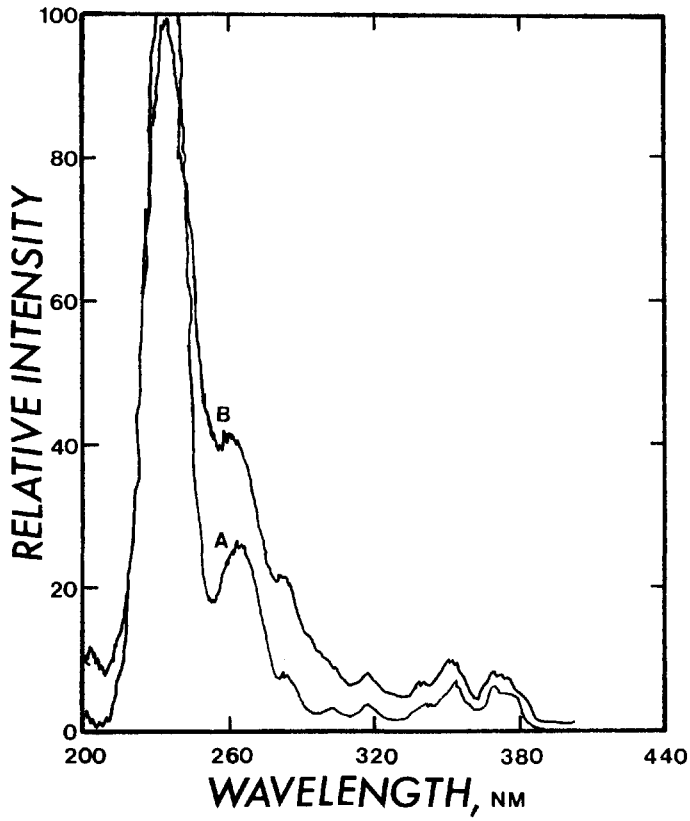

FIGURE 2. Excitation spectra of 2.7 mM terbium (a) and 2.7 mM terbium with 61 μM Ang. II. Emission at 545 nm and pH 7.4.

Lanthanide complexes of amino acids have been synthesized and characterized (Misra et al. 1982) and studied by polarography (Pitre and Chitale 1981), luminescence (Bel'tyukova et al. 1979), titration, and circular dichroism spectroscopy (Prados et al. 1974). Lanthanide ions coordinate the carboxylate group of the amino acids with the ammonium group remaining protonated (pK > 9) and unbound. The zwitterion forms innersphere, monodentate complexes with trivalent lanthanide ions.

The terbium luminescence is enhanced when certain amino acids and small peptides are added to the solution.

TABLE 1

Relative Intensity of Terbium Luminescence in Complexes with Amino Acids and Peptides

Ligand*	Concentration M	Enhancement**
Tyr	5.0×10^{-4}	4.7×10^2
acetyl-Tyr	7.0×10^{-4}	9.7×10^2
Gly-Tyr	5.9×10^{-4}	3.3×10^2
Gly-Gly-Tyr	5.0×10^{-4}	3.2×10^2
Phe	5.0×10^{-4}	23
acetyl-Phe	5.8×10^{-4}	35
Ala	5.0×10^{-4}	0
Trp	5.0×10^{-4}	−89
acetyl-Trp	4.9×10^{-4}	2.3×10^2
Leu	5.0×10^{-4}	0
Ang II	6.1×10^{-5}	1.6×10^2 (280 nm) 45 (259 nm)

* 23 mM Tb (III) and pH 6.5 for all ligands except Ang II which had 2.7 mM Tb (III) and pH 7.4. L-amino acids were used.

**The average formation constant for Eu-Gly and Nd-Ala complexes was used to estimate the concentration of the bound ligand (Tanner and Choppin 1968, Sherry et al. 1973) for the amino acids and small peptides.

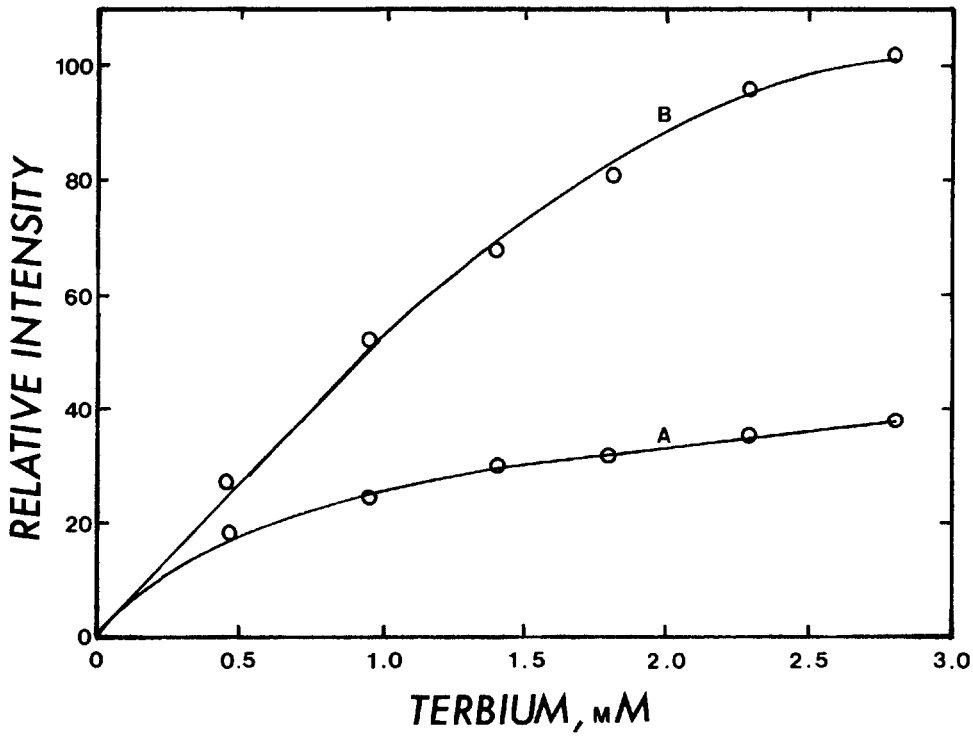

FIGURE 3. Titration of buffer (a) and 61 μM Ang II (b) with terbium excitation at 280 nm, emission at 545 nm and pH 7.4.

Figure 4 illustrates the enhancement for L-tyrosine and n-acetyl-L-tyrosine. The enhancement values are summarized in Table 1. The values reported in Table 1 are different than the values reported earlier (Bel'tyukova et al. 1979). Bel'tyukova et al. (1979) used a Tb^{3+} concentration that was five times greater than that used in our study, and the complexes were subjected to high pH conditions (pH range 7-9). Below pH 7.4, all of our solutions were optically clear. They became cloudy, however, as the pH approached 8, even when a large excess of amino acid was present.

Since the carboxylate group of the zwitterionic amino acids only coordinates weakly to lanthanide ions, methods to increase the binding and terbium luminescence were investigated. First, excess ligand was added to shift the position of equilibrium. However, strong inner filter effects were observed (Martin 1983, pp. 260-264). The use of excess Tb^{3+} provided better experimental conditions. Second, the positive charge associated with the ammonium group was removed from the vicinity of the carboxylate group by peptide bond formation and acetylation. For Gly-Tyr and Gly-Gly-Tyr there was no major change in the enhancement value when compared with the value for Tyr, but the value for acetyl-Tyr increased by a factor of two. The Trp quenched the terbium emission (negative enhancement value), whereas acetyl-Trp gave an enhancement. The observed increase upon actylation is presumably due to a larger formation constant. Attempts to acetylate Ang II in solution were unsuccessful because the excess acetate quenched the terbium luminescence.

Finally, the amino acid results may be used to evaluate the Ang II results. Since experimental results suggest that a Förster dipole-dipole mechanism with an r^{-6} dependence between donor and acceptor metal ion can be used in these systems, Canada (1981) concluded that the low value of 3×10^2 for the enhancement of the Tb-Ang II complex at 280 nm reflects a longer distance between the bound Tb^{3+} and the aromatic side chain of Tyr. Our results show that the distance factor is important but cannot be used to explain the small enhancement factor for the Tb-Ang II complex. When compared to the amino acid data, the enhancement at 259 nm (Phe excitation) is about the same as Phe. In both cases, the carboxylate group of Phe binds directly to the Tb^{3+}. However, the enhancement at 280 nm (Tyr excitation) is only 34% (our value) and 64% (value from Canada 1981) of the value observed for Tyr. The reduction in the enhancement for the Ang II complex is clearly related to the increase in distance between the Tyr^4 donor and Tb^{3+} acceptor. However, the distance factor doesn't account for the large difference in enhancement between the Ang II and protein complexes.

From the data presented in Table 1 and equilibrium constants [$K_d(Asp^1\text{-}COO^-)$ = 11.4 mM and $K_d(Phe^8\text{-}COO^-)$ = 2.8 mM] from mmr studies (Lenkinski and Stephens 1983), we conclude that the low enhancement of the terbium luminescence in Ang II is due to weak binding. The monodentate complexes of Ang II, small peptides, and amino acids do not provide the highly hydrophobic environment found in proteins. Brittian (1980) has investigated the circularly polarized luminescence spectra of mixed amino acid complexes. If the amino acids in these mixed complexes could be selectively excited, a larger enhancement should be observed.

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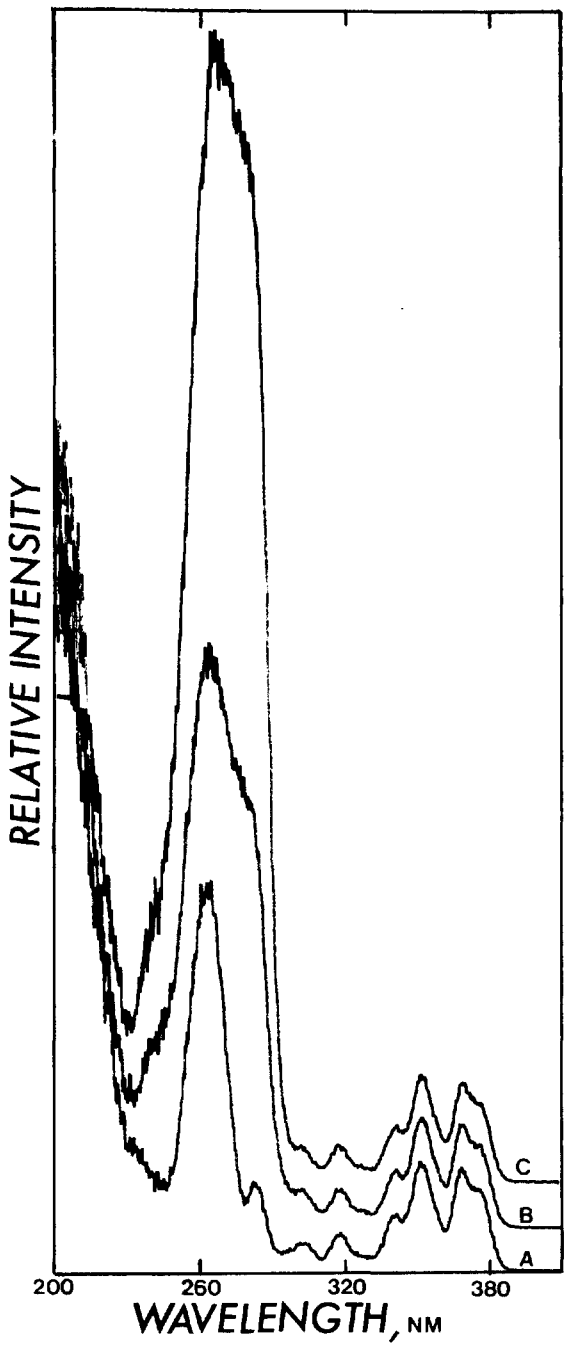

FIGURE 4. Excitation spectra of 23 mM terbium (a), 23 mM terbium with 500 μM tyr (b) and with 700 M acetyl-tyr (c). Emission at 545 nm and pH 6.5. Relative intensity shown in arbitrary units.

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